EXHIBIT 3

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Mapping the Protein Surface of Human Immunodeficiency Virus Type 1 gp120 using Human Monoclonal Antibodies from Phage Display Libraries

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Panels of hybridoma-derived monoclonal antibodies against diverse epitopes are widely used in defining protein surface topography, particularly in the absence of crystal or NMR structural information. Here we show that recombinant monoclonal antibodies from phage display libraries provide a rapid alternative for surface epitope mapping. Diverse epitopes are accessed by presenting antigen to the library in different forms, such as sequential masking of epitopes with existing antibodies or ligands prior to selection and selection on peptides. The approach is illustrated for a recombinant form of the human immunodeficiency virus type 1 (HIV-1) surface glycoprotein gp120 which has been extensively mapped by rodent and human monoclonal antibodies derived by cellular methods. Human recombinant Fab fragments to most of the principal epitopes on gp120 are selected including Fabs to the C1 region, a C1/C5 epitope, a C1/C2 epitope, the V2 loop, the V3 loop and the CD4 binding domain. In addition an epitope linked to residues in the V2 loop and CD4 binding domain is identified. Most of these specificities are associated with epitopes presented poorly on native multimeric envelope, consistent with the notion that these antibodies are associated with immunization by forms of gp120 differing in conformation from that found on whole virus or infected cells.

Keywords: human antibody repertoires; epitope mapping; HIV infection; combinatorial libraries; gp120 topology

Introduction

Some proteins do not yield readily to structural solution by the classical approaches of crystallography or nuclear magnetic resonance (NMR) spectroscopy. The surface glycoprotein gp120 of the human immunodeficiency virus type 1 (HIV-1) is such a protein. Crystallization is hindered by its high carbohydrate content (about 50%) and NMR structural studies by its relatively large size. Still there is an urgent need for structural information on the molecule. For instance such information would be valuable in understanding the nature of the gp120-CD4 and gp120-chemokine receptor interaction which is key to viral entry in to cells. Furthermore the molecule is important in eliciting neutralizing antibodies and so its structure has many implications for vaccine design.

Comparison of gp120 sequences from different HIV-1 strains has identified five variable domains (V1 to V5; Modrow *et al.*, 1987; Starcich *et al.*, 1986), of which the first four form disulphide-stabilized loops, and five conserved domains (C1 to C5). Computer modeling has further been used to suggest the location of secondary structural elements in gp120 (Gallaher *et al.*, 1995). Detailed

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Abbreviations used: HIV-1, human immunodeficiency virus type 1; gp, glycoprotein; Ig, immunoglobulin AP; AP, alkaline phosphatase; CD4bd, CD4 binding domain; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

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information on the tertiary and quaternary structures of the native protein, however, are unavailable.

The surface topography of a protein can be examined through the study of panels of monoclonal antibodies reactive with the protein. Whilst such a study provides a view at far lower resolution than crystallography or NMR it can nevertheless be useful. For example, using a large panel of mostly rodent and some human monoclonal antibodies (mAbs), a low resolution model of gp120 has been constructed (Moore *et al.*, 1993b; Moore *et al.*, 1994a,b; Wyatt *et al.*, 1992; Moore & Sodroski, 1996). This model required the input of antibodies from many laboratories and represents a huge body of work in the generation of the antibodies alone. The antibodies were generated by cellular techniques; rodent mAbs from hybridomas and human mAbs by EBV immortalization of B cells.

Phage display libraries provide a rapid route to large numbers of mAbs from immune donors (Burton & Barbas, 1994; Burton *et al.*, 1991). In the case of recombinant monomeric gp120 as the selecting antigen, antibodies of notable sequence diversity have been retrieved but the great majority are directed to a series of related epitopes on the CD4 binding domain (CD4bd) of gp120 (Barbas *et al.*, 1993). In part this probably reflects the fact that the CD4bd is a major target for serum antibodies to gp120 in HIV-1 seropositive individuals (Moore & Ho, 1995). The relative conservation of this site is likely to be another factor favoring the observed bias to this site since the great variability of HIV-1 means that the libraries are challenged with a gp120 different to the immunizing antigen (we used mostly gp120 from the LAI strain for library selection). Epitopes associated with the variable loops, for example, are less conserved than CD4bd-associated epitopes so it is less likely that Fabs to the variable regions of gp120 will be isolated when heterologous gp120 is used for selection.

If the library approach is to be useful in mapping gp120 topography then other epitopes must be accessed. We have shown that an antibody to the V3 loop can be generated by selection against a constrained peptide corresponding to the crown of the loop (Barbas *et al.*, 1993) and an antibody to a previously undescribed epitope linked to residues in the V2 loop and the CD4bd can be accessed by masking the CD4bd epitope with an existing antibody prior to selection (Ditzel *et al.*, 1995). We show now that further masking can give access to three epitopes associated with the C1 region of gp120 and to an epitope associated with the V2 loop. A linear peptide, corresponding to 24 amino acid residues of the HIV-1 MN V3 loop, is used to select for an antibody to the V3 loop. Therefore, in this model system, the library approach gives access to most of the epitopes described previously on gp120 and suggests the potential utility of the approach in providing antibody reagents for mapping protein surfaces.

Results

Previously, eight HIV-1 libraries have been panned on recombinant gp120 coated directly to microtiter wells which resulted in the isolation of a panel of Fab fragments specific for the gp120 CD4bd. Additional Fab fragments directed against a CD4bd/V2 loop-sensitive epitope have been retrieved after masking of CD4bd epitopes with an anti-CD4bd mAb. To extend the repertoire of human Fabs to a range of other epitopes, we employed a number of different selection strategies.

Epitope masking by capturing the antigen using antibody or ligand

The first strategy employed masking of CD4bd epitopes by capturing gp120 either by soluble CD4 or an anti-gp120 CD4bd mAb immobilized on solid phase. Selection of the libraries on soluble CD4-captured gp120 resulted in the isolation of three novel Fab fragments (Fab p7, p20 and p35). Panning on gp120 captured by the anti-CD4bd mAb yielded ten additional Fab fragments (Fab L15, L17, L19, L25, L34, L35, L52, L59, L69 and L81). The specificity of the different Fab fragments was demonstrated by their strong ELISA reactivity with gp120, but not with ovalbumin, human Fc fragment, transferrin or bovine serum albumin (BSA). The 13 Fab fragments were demonstrated to be diverse by sequence analysis of the variable regions of the heavy and light chains. As shown in Figure 1, the sequences of the heavy chain CDR3s were unrelated except for Fab L59 and L69, for which the whole heavy chain variable domain sequences differed by only seven amino acid residues from one another and which therefore may be somatic variants.

To determine which epitopes are recognized by the Fabs, we assessed their binding to a panel of HXBc2 gp120 mutants expressed in COS-1 cells. Binding of Fab p7, p20 and p35 revealed that all three Fabs are directed to closely related epitopes located in the N-terminal region of gp120. As shown in Figure 2(a), the binding of Fab p7 was completely abolished by amino acid substitution 45 W/S in the C1 region. Binding of Fab p7 was further markedly reduced by amino acid substitution 40 Y/D, and showed some dependency on substitutions at the C terminus of gp120, as demonstrated by decreased or enhanced binding by substitutions 475 M/S and 493 P/K. Very similar mutant maps were found for Fabs p20 and p35 (not shown).

The Fabs selected on gp120 captured by the anti-CD4bd mAb recognize four distinct epitope clusters. The majority of Fabs (i.e. L19, L34, L35, L52, L59, and L69) recognize a C1 epitope very similar to that recognized by Fabs p7, p20 and p35, as described above. A second epitope involving the C1 and C5 regions is recognized by Fab L81 (Figure 2). The binding of Fab L81 is abolished by a substitution in the C1 region (45 W/S)

Fab	FR3	CDR3	FR4
L15	TAVYYCAR	DSPGYSNTWYDWFEP	WGQGTLVTVSS
L17	TAVYYCAT	GRPRWWQRDAFHF	WGQGTKVTVSS
L19	TAVYYCAR	HSGRYINGNYHPYGMDV	WGQGTTVTVSS
L25	DARYYCAR	AWEVRIDHRYFFDL	WGQGTLVTVSS
L34	TAVYYCAR	QPLARHFDP	WGQGTLVTVSS
L35	TAIYYCAS	PLYPPKGPIVATTDY	WGQGTLLTVSS
L52	TAVYYCAR	GCQHLVNYFDY	WGQGTLVTVSS
L59	TAVYFCAR	DNGLPHNHFDP	WGQGTQVTVSS
L69	TAVYYCAR	DKGLPYNHFDP	WGQGTQVTVSS
L81	TALYYCAK	EGEQVGYFDWRTKLRFSFFDL	WGRGTLVTVSS
p7	TAVYYCAT	DGSRLSTSAFDFWSGNRPSSYIDV	WGKGTAVTVSS
p20	TAVYYCAR	RLIGGTFPFFRYSYVDV	WGTGTTVTVSS
p35	TAVYYCAR	DQGIRVAGGLDY	WGQGTLVTVSS
L100	TAIYYCAK	GPLMRWFDD	WGQGTLVAVSS
DO142-10	TAIYYCAR	SHCGSNCYGLFEH	WGQGTLVTVSS

Figure 1. Amino acid sequences of the heavy chain CDR3 region and adjacent framework regions of anti-gp120 Fabs.

and is also abolished by a mutation in C5 (491 I/F), and is strongly impaired by a substitution in C3 (349 L/A).

A third epitope group of two Fabs (Fab L15 and L17) isolated by selection on ant-CD4bd mAb-captured gp120 is specific for the V2 loop. As shown in Figure 2, substitutions in or deletion of the V1/V2 loops abolished binding of Fab L15 to gp120. Fab L15 further competed with rodent anti-V2 mAbs SC258 (Figure 3(a)), CRA3, G3-4, G3-136, BAT-085 and 52-684 (data not shown) but not with anti-gp120 mAbs directed to other epitopes (Figure 3(a)). The binding pattern of Fab L17 is very similar to that observed for Fab L15 (not shown).

The fourth epitope group obtained by CD4bd-captured gp120 selection consists of the single antibody, Fab L25. The binding of this antibody to the panel of gp120 mutants demonstrated a sensitivity to substitutions in residues associated with the CD4 binding site and the V2 loop (Figure 2) as also observed for three previously described human Fabs (Ditzel *et al.*, 1995). Furthermore, Fab L25 competed for binding to gp120 with murine anti-V2 mAb SC258 (Figure 3(a)). This Fab is therefore directed to an epitope which we have termed the CD4bd/V2 loop-sensitive epitope.

Multiple epitope masking

To demonstrate the ability to sequentially mask epitopes on an antigen to retrieve antibodies with new specificities, CD4bd mAb-captured gp120 was masked with one of the high-affinity anti-C1 region antibodies, Fab p7, prior to addition of phage. After selecting the pooled phage-display libraries for four rounds, two gp120 specific Fab fragments were isolated. Heavy chain sequence analysis identified one as anti-V2 loop Fab L17. The other represented a novel antibody, Fab L100 (Figure 1), which was found to bind to an epitope involving the C1 and C2 regions (Figure 2). Substitutions 69 W/L and 76 P/Y abolish the binding of Fab L100 implying that the antibody binds to a part of the C1 region distinct from that recognized by the masking antibody, Fab p7. Substitutions 252 R/W, 256 S/Y, 262 N/T and 267 E/L abolish or strongly impair the binding of Fab L100, indicating direct or indirect involvement of the C2 region in the epitope recognized.

Selection on peptides

A different approach to retrieve antibodies directed against distinct epitopes on an antigen is to use constrained or linear peptides. We have used this approach in attempts to isolate antibodies to the V3 and V1 loops of gp120. With respect to the V3 loop, libraries were panned against four different peptides: (1) a linear peptide corresponding to 24 residues of the HIV-1 MN V3 loop (RP142) coupled to ovalbumin; (2) a linear 24 residue peptide corresponding to the HIV-1 LAI V3 loop (RP135); (3) a cyclic peptide (N = CH-(CH2)3CO[-SISGPGRAFYTG]NH2CO-Cys-NH2) corresponding to the central most conserved part of the clade B V3 loop coupled to BSA, and (4) a linear peptide corresponding to the "consensus " clade B V3 loop (PND peptide). Two neutralizing Fab fragments were selected. The first anti-V3 antibody, Fab loop 2, obtained by panning against the cyclic V3 loop peptide has been described (Barbas *et al.*, 1993). Fab loop 2 recognizes gp120 from HIV-1 strains MN and SF2 but not LAI. The second antibody, Fab DO142-10, was selected by panning against the RP142 peptide. The same Fab was also retrieved by panning against recombinant gp120 MN. To probe antigen specificity, Fab DO142-10 was tested for binding against a number of V3 loop peptides, recombinant gp120 proteins and unrelated antigens. As shown in Figure 4(a), Fab DO142-10 bound equally well to the RP142 peptide and gp120 MN, which were both used as selecting antigens. Lower binding affinities were observed with the PND peptide, a JR-CSF V3 loop fusion protein and a recombinant gp120 from primary isolate W61D. Fab DO142-10 did not bind appreciably to recombinant gp120 LAI or to a panel of unrelated antigens which included BSA, the Fc fragment of IgG and ovalbumin. Fab DO142-10

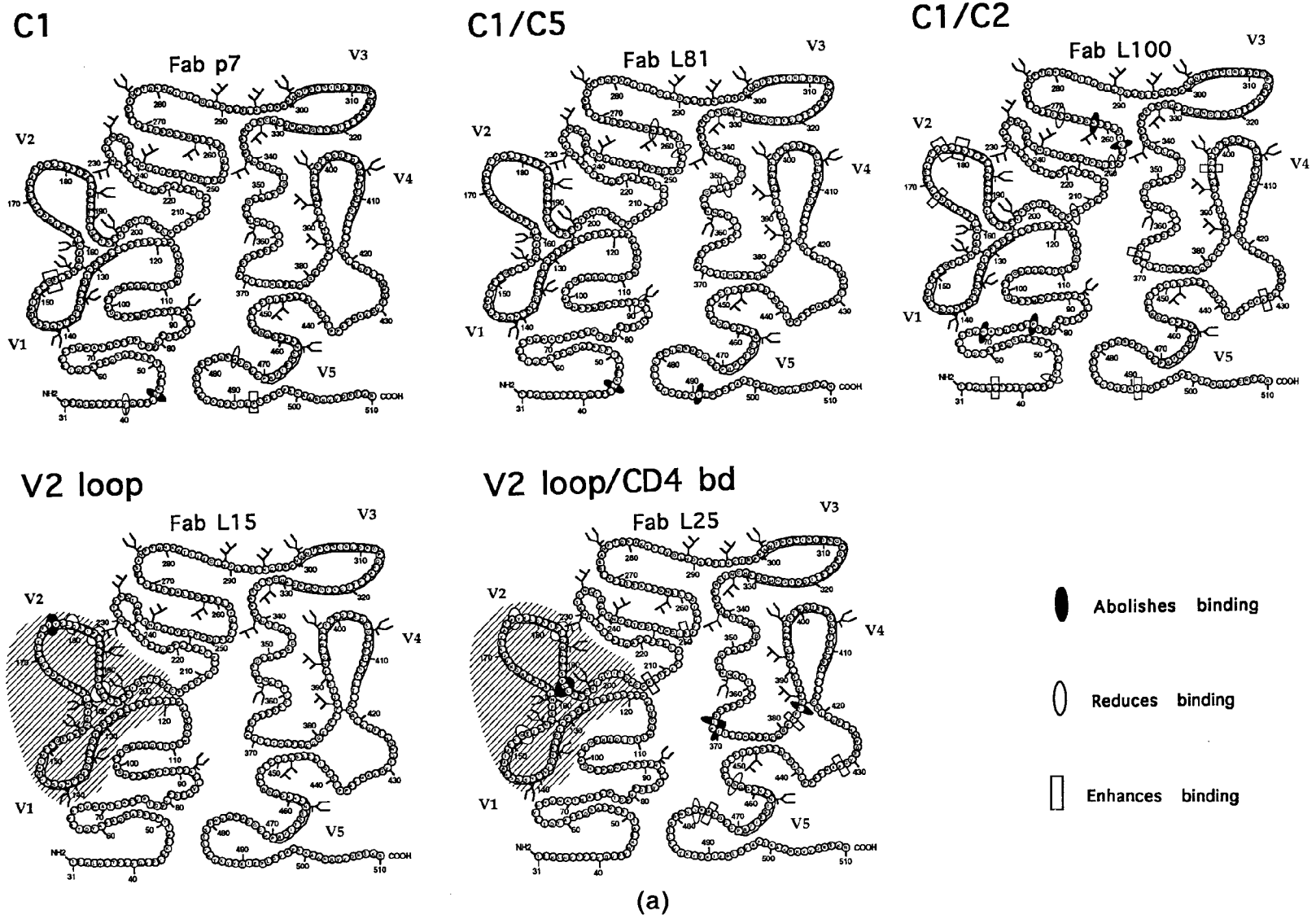

Figure 2(a) (*legend on page 688*)

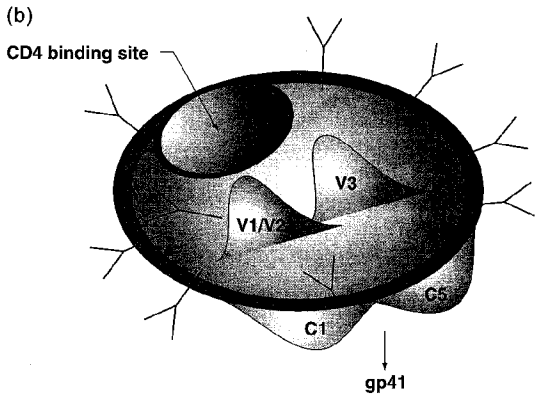

Figure 2(b)

Figure 2. (a) Relative binding of human recombinant Fabs to selected HIV-1 LAI gp120 (HXBc2) mutants. The primary sequence of gp120 is shown as a disulphide map. The position of putative N-linked carbohydrate attachment sites are indicated schematically. Only mutations abolishing binding (closed ovals; binding ratio <0.2), decreasing binding (open ovals; binding ratio <0.5) or enhancing binding (open rectangles; binding ratio >2.0) of Fabs are indicated by superposition of oval or rectangle symbols on the primary sequence. The hatched region including V1 and V2 loops in the bottom two maps indicates strong impairment of Fab binding upon deletion of these loops. Mutations at positions that did not affect the binding of the Fabs shown are not indicated. Fab binding to the gp120 mutant panel was repeated three times with similar results. The following mutants were included in the panel tested: 36V/L, 40Y/D, 45W/S, 69W/L, 76P/Y, 80N/R, 88N/P, 102E/L, 103Q/F, 106E/A, 113D/A, 113D/R, 117K/W, 120/121VK/LE, 125L/G, Δ119-205 (V2), 152/153GE/SM, 168K/L, 176/177 FY/AT, 179/180LD/DL, 191/193YSL/GSS, 207K/W, 252R/W, 256S/Y, 257T/R, 262N/T, 166A/E, 267E/L, 169E/L, 281A/V, 298R/G, 313P/S, 314G/W, 356N/I, 368D/R, 368D/T, 370E/R, 370E/Q, 380G/F, 381E/P, 384Y/E, 386N/Q, 392N/E + 397N/E, 395W/S, 406N/G, 420I/R, 421K/L, 427W/V, 427W/S, 429K/L, 430V/S, 432K/A, 433K/A, 435Y/H, 435Y/S, 438P/R, 456R/K, 457D/A, 457D/R, 463N/D, 470P/L, 470P/G, 475M/S, 477D/V, 485K/V, 491I/F, 493P/K, 495G/K, 500/501 KA/KG. (b) A schematic model for gp120 structure. The diagram was adapted from Burton & Montefiori (1997), Sodroski *et al.* (1996), and Poignard *et al.* (1996). Extensive glycosylation is schematically indicated by Y-shaped protrusions. The C1 and C5 regions are accessible on monomeric gp120 but buried on native gp120 on the viral surface, probably by interaction with the transmembrane envelope glycoprotein gp41. gp120-gp41 heterodimers are present in the form of oligomers, probably trimers, on the cell or virion surface. The V1/V2, V3 regions and the CD4 binding site are accessible to antibody on monomeric gp120. These antigenic sites are less accessible, however, on gp120 in oligomeric configuration on the envelope of T-cell line adapted HIV-1, and exposure of the V3 region and CD4 binding site in particular are highly restricted on the envelope of primary viruses.

was also tested for binding to gp120 captured from a set of viral culture supernates which included HIV-1 AD6, JR-FL, Al-1, MN, SF2, 146, NYC-1, 120, and 437. Strong binding was observed to gp120 from HIV-1 MN and JR-FL, lesser binding to SF2 gp120 and no binding to the other gp120s (Figure 4(b)).

With respect to the V1 loop of gp120, we panned selected libraries against four linear 26-mer peptides corresponding to the sequences of the V1 region of gp120 from LAI and three primary isolates (case B, RA and VS). Serum titers of all eight library donors to the peptides were weak (<1:50), and no V1-specific clones were isolated from the human libraries or from a library prepared from bone marrow of a chimpanzee infected with HIV-1 LAI.

Further characterization of the N-terminal (C1) reactive Fab fragments

The selection experiments yielded Fabs to more than one epitope involving the C1 region. These Fabs were not retrieved by selection on gp120 directly coated to the solid phase. We decided to investigate these Fabs in more detail. The Fabs were cross-competed with a panel of rodent and human mAbs which included mAb M85 directed against a linear epitope in the extreme N-terminal region of gp120, mAbs M90 and 212A directed to different conformational epitopes in the N-terminal region, mAb M91 directed to a linear epitope in the C5 C-terminal region, and mAb G3-299 directed to the C4/V3 region (Figure 3(b)). The Fabs isolated by selection on soluble CD4-captured gp120 (Fab p7, p20 and p35) and mapped to a C1 region epitope competed with mAbs M85, M90 and 212A, but not mAbs M91 and G3-299. Fabs L52, L69, L34 and L35 retrieved by CD4bd antibody-captured gp120 showed a similar competition pattern as the p7 group. Fab L19 exhibited a broadly similar pattern but showed some inhibition of mAbs M91 and G3-299 and inhibited mAb M90 less efficiently. Binding of Fab L81, which was mapped to a C1/C5 region epitope, was inhibited by N-terminal reactive antibodies and mAb M91 directed against a C-terminal gp120 epitope. Fab L100 directed against the C1/C2 region did inhibit mAb M90 efficiently but not mAbs M85, 212A and M91. Some inhibition of the anti-C4 antibody G3-299 was also observed. The antibody inhibition studies are therefore consistent with the mutant mapping studies in identifying Fabs recognizing three distinct epitopes all involving the N terminus of gp120.

To elucidate why the extensive panel of C1 region reactive Fabs was not retrieved by selection with directly immobilized gp120, the Fabs were tested for binding to gp120 either coated to microtiter wells or captured by a sheep antibody (D7324) to the extreme C terminus of gp120. Interestingly, the C1 region reactive Fab fragments p7, p20, p35 and L19 did not bind to gp120 coated directly on the microtiter well (Figure 5(a)) (Fab p20

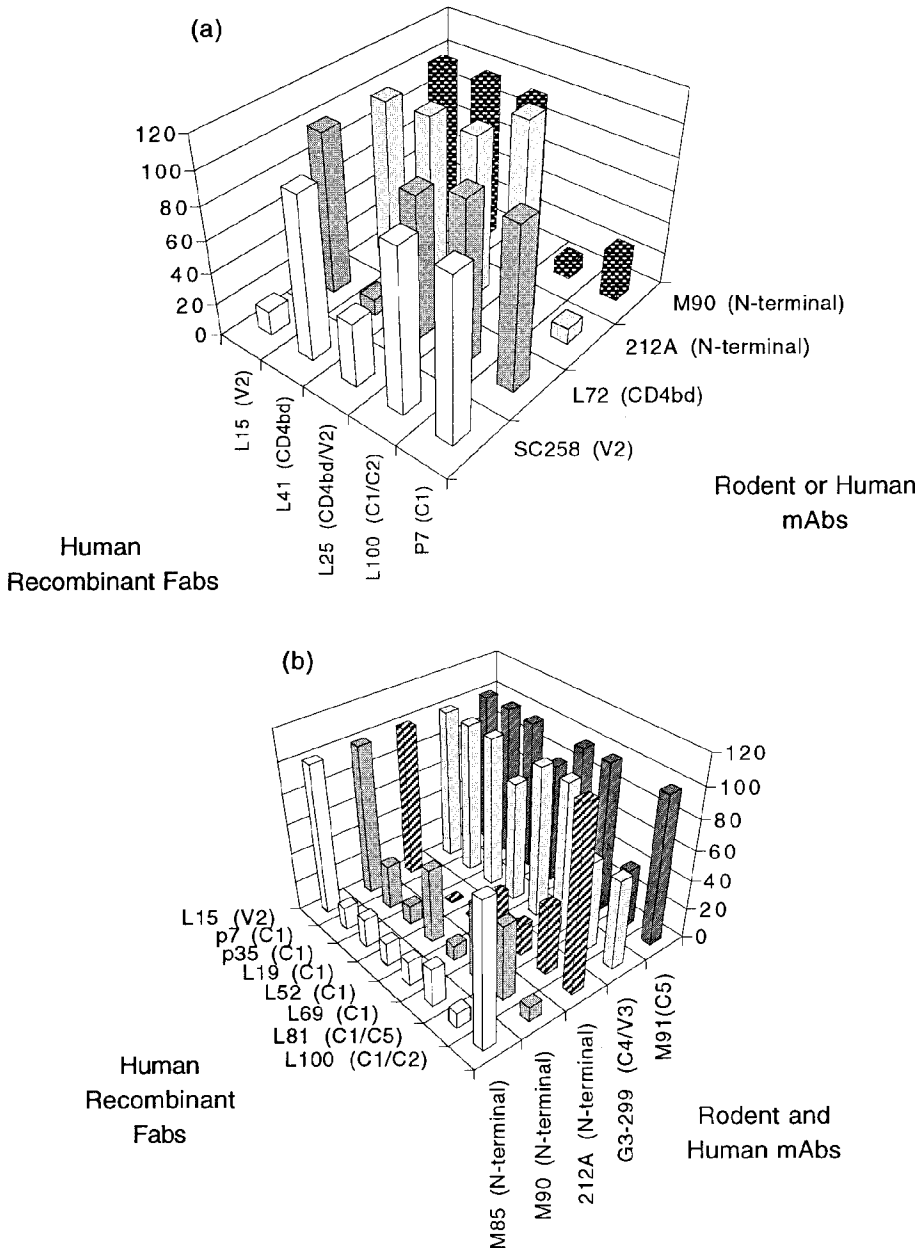

Figure 3. Competition between Fabs (a) directed to a diverse set of epitopes and (b) directed to overlapping N-terminal epitopes, with panels of rodent or human anti-gp120 mAbs. The epitope specificity of the human or rodent antibodies is specified between parenthesis. Bound rodent or human mAb (in a concentration giving 75% maximum binding) to gp120 LAI competed with the human Fab fragments at a concentration 100 times that giving 75% maximum binding in previous titration experiments was detected with AP-labeled anti-mouse IgG, anti-rat or anti-human IgG Fc antibody. Binding is expressed in %, with the A_{405} of uncompeted antibody set as 100%.

and p35 not shown). Other Fabs mapped to C1, such as Fab L69, in contrast, bound to gp120 in both assay formats, although the binding to captured gp120 was considerably stronger (Figure 5 (a and b). CD4bd and V2 loop Fabs bound similarly to gp120 in both formats. This indicates that gp120 directly immobilized on to a microtiter well coats in an orientation which occludes part of the N-terminal region of the molecule.

We further examined whether Fab p7, p20 and p35 had higher affinity for captured gp120-CD4 complexes as compared to uncomplexed gp120. The Fabs bound efficiently in both cases. However, a small but significant increase in binding was seen to the CD4-complexed gp120, especially for Fabs p20 and p35 (data not shown).

To determine if the anti-C1 region Fabs recognized linear epitopes on gp120, gp120 was de-

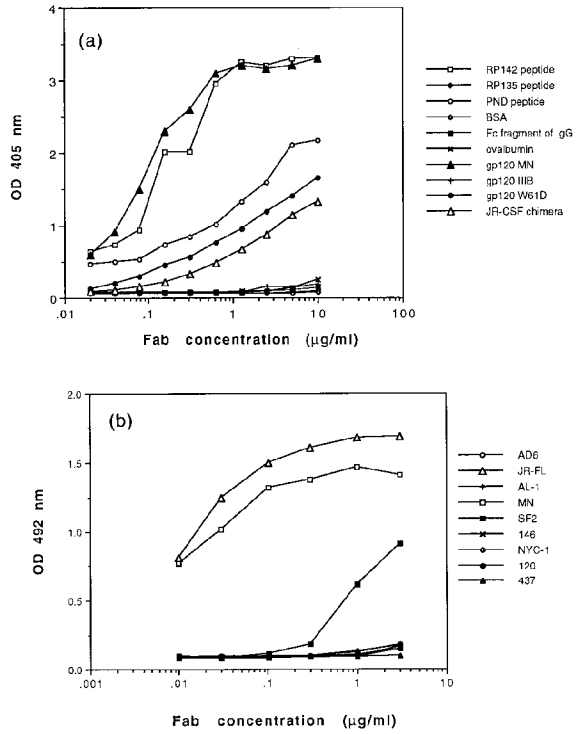

Figure 4. Binding of the anti-V3 region Fab DO142-10 to: a, a panel of V3 peptides, recombinant gp120 proteins and unrelated antigens; b, gp120 captured from a panel of HIV-1 culture supernates using a polyclonal sheep antibody to the C terminus of gp120.

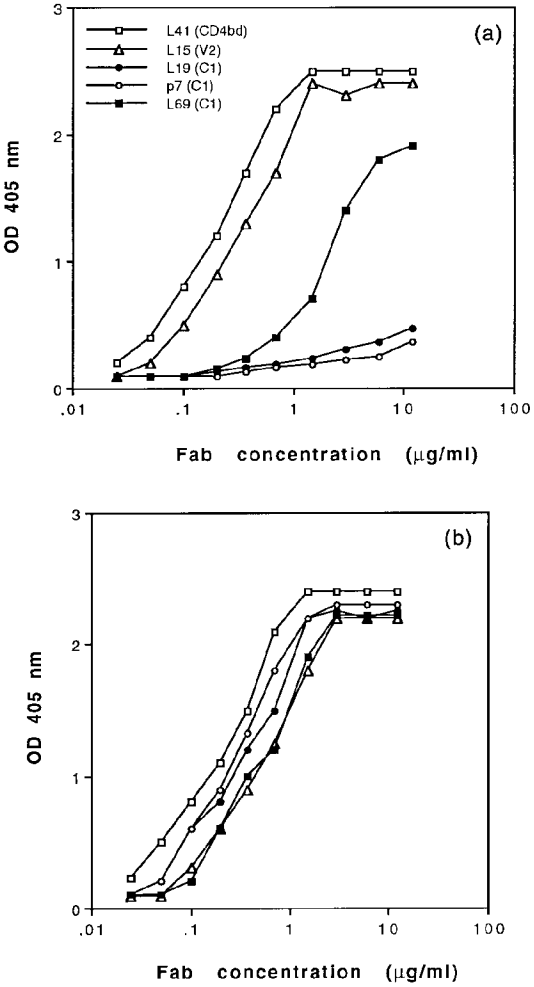

Figure 5. Binding of recombinant Fabs to: a, recombinant gp120 LAI directly coated on microtiter wells; or b, gp120 LAI captured by a polyclonal sheep antibody to the extreme C-terminus of gp120.

natured and captured by either anti-V3 mAb D47 or polyclonal antibody D7324. As shown in Figure 6, the binding of Fab p7 was reduced by denaturation but not completely abrogated. Similar observations were found for the other anti-C1 Fabs. As a control, antibody IIIB-V3-13, recognizing a linear epitope in the V3 region, was run in parallel and was found to bind approximately equivalently to both native and denatured gp120 (data not shown) as reported (Moore *et al.*, 1994a).

Fab affinity for recombinant gp120

The binding of a panel of selected Fabs to recombinant HIV-1 gp120 LAI and MN was measured by surface plasmon resonance using the BIAcore as shown in Table 1. Fabs p7 and L19 exhibited very weak binding to sensor chip-immobilized HIV-1 LAI gp120 and binding kinetics were therefore measured with an HIV-1 LAI gp140 oligomer. Affinities for all the studied Fabs were in the range of 3×10^7 to 4×10^9 M^{-1}.

Virus neutralization

Purified Fabs from each of the clones in the panel were initially examined for neutralizing ability in infectivity assays employing the MN and LAI strains of HIV-1. Neutralization was determined as the ability of the Fab fragments to inhibit infection, as measured by a plaque reduction assay using MT-2 cells and syncytium formation using CEM-SS cells. In both assays none of the Fab fragments mapped to epitopes involving C1, including Fab L100, were neutralizing (data not shown). Anti-V2 loop Fab L15 also exhibited no neutralization. The CD4bd/V2 loop-sensitive Fab L25 showed only weak neutralization (data not shown). In contrast, in the plaque reduction assay, the syncytium inhibition assay and in an envelope-complementation neutralization assay, Fab DO142-10 demonstrated potent neutralization of HIV-1 MN with 50% neutralization titers at 0.2, 7 and 2 μg/ml, respectively. Weaker neutralization was observed against HIV-1 LAI, with 50% neutralization titers at 6, 8 and 8 μg/ml, respectively. The other V3 loop antibody, Fab loop 2, also potently neutralized HIV-1 MN with

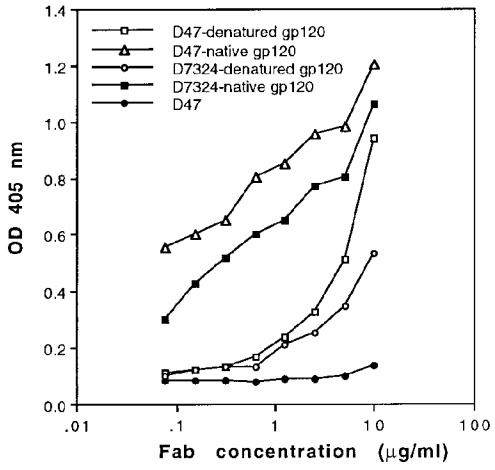

Figure 6. Binding curves of Fab p7 (anti-gp120 C1 region) to native and denatured gp120 LAI captured on polyclonal anti-C terminal anti-gp120 antibody D7324 or anti-V3 loop mAb D47.

50% neutralization titers at 1, 5 and 2 μg/ml, respectively. Fab loop 2 did not neutralize HIV-1 LAI.

Discussion

Construction and selection of antibody phage display libraries offers an efficient route to obtain mAbs, including human antibodies (Burton & Barbas, 1994; Winter *et al.*, 1994). Considerable efforts have focussed on creating larger and more diverse naive or synthetic libraries where most reports describe the isolation of one or two antibodies to a given antigen. For some applications, including protein mapping, however, it may be essential to obtain antibodies to a range of epitopes. This may require more than simple selection of the library against the antigen of interest. Here we describe selection procedures leading to the isolation of an extended set of specificities to a single antigen. The antigen investigated is the HIV-1 surface glycoprotein gp120, which is a well characterized molecule with several advantages for this study. These include the availability of many rodent and human mAbs to the molecule, synthetic peptides corresponding to linear epitopes of the molecule and an extensive set of mutant molecules. All of these resources assist in demonstrating the specificities of the antibodies retrieved from libraries. The approach described, however, should be generally applicable even in the absence of such resources.

Selection of HIV-1 immune libraries against recombinant gp120 yields overwhelmingly antibodies reactive with the CD4bd (Barbas *et al.*, 1993). One strategy to refocus selection was to capture gp120 on soluble CD4. This resulted in selection of Fabs reactive with an epitope overlapping the N-terminal (C1) region of gp120, a site which was shown by ELISA to be mostly occluded on gp120 coated directly onto plastic. These antibodies did bind to gp120 in the absence of soluble CD4, although some moderate enhancement of binding in the presence of the ligand was observed. This enhancement was less than that observed with the mAbs 17b and 48d, which are often described as binding to a "CD4-induced " epitope (Thali *et al.*, 1993). Mapping on gp120 mutants shows no evidence for the involvement of N-terminal residues in binding of the mAbs 17b or 48d.

Refocussed selection was also achieved using an anti-CD4bd mAb to capture gp120. The selected antibodies included several C1 region reactive Fabs as described above (p7 group of Fabs) but also another related epitope recognized by Fab L81 which involved residues from the N terminus (C1 region) but also residues from the C terminus (C5 region). A specificity for C5 was indicated by mutant binding analysis and competition with a murine mAb to the extreme C terminus. These results provide further evidence for the proximity of the C1 and C5 regions (Moore *et al.*, 1994b). Selection against CD4bd mAb-captured gp120 also yielded Fabs specific for the V2 loop and Fabs against a novel epitope which we have described as CD4bd/V2 loop-sensitive (Ditzel *et al.*, 1995). This epitope is not accessed by CD4-captured gp120 since it appears that the epitope is occluded on CD4 binding. Anti-CD4bd antibodies, in contrast,

Table 1. Kinetic constants and affinity constants for the binding of selected Fabs to gp120 measured by surface plasmon resonance

Fab	$k_{on}(M^{-1}s^{-1})$	$k_{off}(s^{-1})$	$K_a(M^{-1})$	$K_d(M)$
b12	1.1×10^5	5.2×10^{-4}	2.1×10^8	4.7×10^{-9}
L17	1.9×10^4	1.9×10^{-4}	1.0×10^8	1.0×10^{-8}
L15	1.1×10^4	3.9×10^{-4}	2.8×10^7	3.5×10^{-8}
L19	1.3×10^5	3.6×10^{-5}	3.6×10^9	2.8×10^{-10}
L69	5.5×10^4	5.1×10^{-4}	1.0×10^8	1.0×10^{-8}
p7	1.5×10^5	1.0×10^{-4}	1.5×10^9	6.8×10^{-10}
DO142-10	1.6×10^4	1.8×10^{-4}	8.9×10^7	1.1×10^{-8}
Loop 2	1.2×10^4	2.3×10^{-5}	5.2×10^8	1.9×10^{-9}

Kinetic constants were measured with gp120 LAI for Fabs b12, L17, L15, and L69; with gp120 MN for Fabs DO142-10 and loop 2; and with a gp140 LAI oligomer for Fabs L19 and p7. The equilibrium association and dissociation constants were calculated from the experimentally determined kinetic constants with $K_a = k_{on}/k_{off}$ and $K_d = k_{off}/k_{on}$.

enhance expression of this epitope. The observation that conformational changes occur upon antigen-ligand interaction, which may not be mimicked by antibodies to the ligand binding site, may be a feature of other antigen systems and should be borne in mind when developing selection strategies for phage-display libraries.

The strategy of refocussed selection was further extended by masking the gp120 N terminus on the anti-CD4bd-captured gp120 by one of the C1 region reactive Fabs. This resulted in the selection of a Fab which recognizes a novel conformational epitope involving the C1 and C2 regions.

In only one instance did we select antibodies against the V3 loop by selection against gp120. This is despite the original designation of the V3 loop as the "principal neutralizing domain " of gp120. Several factors likely contribute to this paucity. First the human response to V3 during natural infection is probably less than that of mice immunized with recombinant gp120. Second we selected with gp120 of a single strain (mostly LAI) which almost certainly differed, particularly in V3, from the eliciting strain in the donor at the time of marrow collection for library construction. Third, because of variability the antibodies against V3 may be of lower affinity against "consensus " sequences than antibodies against conserved regions such as the CD4bd. Phage selection strongly favors clones of higher affinity (Barbas *et al.*, 1991) and so weakly cross-reactive anti-V3 clones may be lost during selection. The use of peptides can be useful in selecting antibodies reactive with largely continuous epitopes, as shown by the retrieval of two anti-V3 loop antibodies using V3 peptides.

The overwhelming majority of antibodies selected in this study showed poor or no neutralization of even T-cell line adapted strains of HIV-1 (primary isolates would probably be yet more difficult to neutralize (Burton *et al.*, 1994; Moore *et al.*, 1995)). Poor neutralization correlates with low reactivity with native multimeric gp120 on cell surfaces (Roben *et al.*, 1994; Sattentau & Moore, 1995), an extreme example being C1/C5 epitope antibodies which show no reactivity with cell surface envelope (Sattentau & Moore, 1995). However, the affinities of these antibodies for recombinant monomeric gp120 are shown by surface plasmon resonance studies to be high, suggesting they do result from antigen-driven processes. We suggest that this antigen is viral debris e.g. gp160 or shed gp120 generated during rapid viral turn-over (Ho *et al.*, 1995; Wei *et al.*, 1995) and not native virions. The antibody response to native virions may in fact be very limited.

Materials and Methods

Library construction and phage selection

Preparation of RNA from bone marrow lymphocytes and subsequent construction of IgG1 κ/λ Fab libraries using the pComb3 M13 surface display system has been described (Barbas *et al.*, 1991; Burton *et al.*, 1991; Persson *et al.*, 1991). For antibody selection, phage libraries generated from eight different HIV-1 seropositive donors were panned separately for the initial round and, after this, pooled together and panned additional rounds. The eight asymptomatic HIV-1 seropositive donors from whom bone marrow was aspirated for library construction have been described elsewhere (Ditzel *et al.*, 1994). Panning of the combinatorial libraries was carried out as described, with slight modifications (Burton *et al.*, 1991; Ditzel *et al.*, 1995). Baculovirus-expressed recombinant gp120, LAI strain (gp120 BRU, Intracel, Cambridge, MA) (0.1 μg/well) in PBS (phosphate-buffered saline (pH 7.4)) was captured by recombinant soluble CD4 (AIDS Research and Reference Reagent Program, Division of AIDS, NIH) (5 μg/ml) or a mouse anti-gp120 CD4bd mAb (mAb L72, kindly provided by Dr Hariharam, IDEC Pharmaceuticals Corporation, La Jolla, CA (Kang *et al.*, 1994)). In other panning experiments the following peptides or peptide-protein complexes were coated directly on ELISA wells: (1) a linear peptide corresponding to 24 residues of the HIV-1 MN V3 loop (RP142) (Repligen, Cambridge, MA) coupled to ovalbumin by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) conjugation (Pierce); (2) a linear peptide corresponding to 24 residues of the HIV-1 LAI V3 loop (RP135) (Repligen) coupled to ovalbumin; (3) a cyclic peptide N = CH-(CH2)3CO[SISGPGRAFYTG]NH2CO-Cys-NH2 corresponding to the central most conserved part of the clade B V3 loop coupled to BSA; (4) a linear peptide corresponding to a "consensus " clade B V3 loop ("principal neutralizing domain " (PND) peptide); (5) four linear 26 amino acid residue peptides corresponding to the sequences of the V1 loop of gp120 from HIV-1 LAI and three HIV-1 primary isolates: case B, RA and VS (kindly provided by Seth Pincus).

ELISA analysis

The human Fabs were purified from bacterial supernatants by column affinity chromatography using immobilized chicken anti-human Fab fragment. To assess specificity, supernatants were screened against gp120 and a panel of control antigens, which included BSA, ovalbumin, and the Fc fragment of human IgG (Sigma, St Louis, MO) by ELISA. Coating of ELISA wells was carried out as described (Ditzel *et al.*, 1995). Fabs were incubated with test antigen for two hours at 37°C, followed by washing ten times with PBS, 0.05% (v/v) Tween. Detection of bound Fabs was carried out with alkaline phosphatase (AP)-labeled goat anti-human IgG $F(ab')_2$ (Pierce, Rockford, IL) diluted 1:500 in PBS and developed with nitrophenol substrate (Sigma). Absorbance was read at 405 nm. To investigate if the epitopes recognized by the Fab fragments were conformational, gp120 was denatured and reduced by boiling for five minutes in PBS containing 1% (w/v) sodium dodecyl sulfate (SDS) and 50 mM dithiothreitol (DTT) before ten-fold dilution into PBS containing 1% (v/v) NP40 to the concentration used (0.1 μg/well) (Moore & Ho, 1993). Native or denatured gp120 was then captured on a solid phase *via* the carboxy terminus using sheep polyclonal antibody D7324 (Aalto Bioreagents, Dublin, Ireland). A murine mAb IIIB-V3-13 (Laman *et al.*, 1992), which has been shown to react almost as well with denatured gp120 as with the native molecule, was used as a positive control. Binding of Fab DO142-10 to gp120 from a panel of different HIV-1 isolates was assessed by capture of gp120 from infected cell lysates as described elsewhere (Trkola *et al.*, 1995).

Nucleic acid sequencing

Nucleic acid sequencing was carried out on a 373A automated DNA sequencer (ABI, Foster City, CA) using a *Taq* fluorescent dideoxy terminator cycle sequencing kit (ABI). Sequencing primers were as reported (Ditzel *et al.*, 1994). The DNA sequences of the Fab heavy chains are accessible in Genbank under the following numbers (if two are given, the second number refers to the light chain sequence): DO142-10: U82961, U82962; L15: U82942; L17: U82943; L19: U82944; L25: U82945; L34: U82946; L35: U82947; L52: U82948; L59: U82949; L69: U82950; L81: U82951; L100: U82952; p7: U82767, U82768; p20: U82769, U82770; p35: U82771, U82772;

CD4 and V1/V2 competition ELISAs

Recombinant gp120 was coated overnight at 4°C onto ELISA wells and blocked with 3% (w/v) BSA for one hour. Soluble CD4 in a tenfold dilution series (10^{-12} to 10^{-6}M) or serial dilution of a HXB2d fusion protein (kindly provided by Abe Pinter) at a concentration of approximately 1 to 10 μg/ml was added together with Fab at a fixed concentration, previously determined to give 75% of maximum binding and incubated for two hours at 37°C. Following washing with PBS-Tween, bound human Fab was detected with AP-labeled goat-anti-human IgG $F(ab')_2$ and developed as described above. A HXB2d V3 fusion protein was used as a control antigen for the V1/V2 competition (Kayman *et al.*, 1994)

Surface plasmon resonance to measure Fab binding affinities

The kinetics of Fab binding to recombinant LAI gp120 and a recombinant LAI gp140 preparation (Earl *et al.*, 1994) were determined by surface plasmon resonance using BIAcore (Parren *et al.*, 1996). Coupling of recombinant gp120 and gp140 to the sensor chip and subsequent binding of the Fab fragment to the immobilized antigens were performed as described (Binley *et al.*, 1996). The association and dissociation rate constants, k_{on} and k_{off}, were determined as described (Karlsson *et al.*, 1991). Equilibrium association and dissociation constants were deduced from the rate constants.

Epitope mapping by antibody cross-competition

Cross-competition experiments were performed between recombinant Fab fragments and a panel of murine and human mAbs. These included anti-C1 region mAbs: M85, M90, and M91 (kindly provided by Fulvia di Marco-Veronese) (di Marzo Veronese *et al.*, 1992); anti-V2 mAbs: 52-581-SC258 (SC258), 52-684-238 (52-684) (Moore *et al.*, 1993a) (kindly provided by Gerry Robey), CRA-3 (MRC AIDS Reagent Project, Potters Bar, Herts, UK), G3-4, G3-136, and BAT-085 (kindly provided by David Ho) (Fung *et al.*, 1992; Ho *et al.*, 1991; Sullivan *et al.*, 1993); N-terminal region mAb 212A (kindly provided by Jim Robinson); anti-C4 MAb G3-299 (kindly provided by David Ho) (Moore & Sodroski, 1996); and anti-V3 loops mAbs IIIB-V3-13 (AIDS Research and Reference Reagent Program, NIH) (Laman *et al.*, 1992) and D47 (kindly provided by Pat Earl) (Earl *et al.*, 1994).

Coating of gp120 onto microtiter wells was carried out as described (Burton *et al.*, 1991). Competing antibody at large excess (a concentration 100 times that giving 75% maximum binding in previous titration experiments) was incubated with the human Fab for two hours. Following washing, bound human Fab was detected, as described above. The assay was also reversed so that the human Fab was added at large excess (a concentration 100 times that giving 75% maximum binding in previous titration experiments). The murine antibody was detected with an AP-labeled goat-anti-mouse IgG (Pierce). Controls without competing antibody and with irrelevant antibody were included.

Epitope mapping of Fabs by binding to gp120 mutants

For mapping of the binding site for the human Fabs, COS-1 cell expressed wild-type or mutant HIV-1 (HXBc2) envelope glycoproteins were captured onto solid phase using sheep-anti-gp120 antibody D7324, as described elsewhere (Moore *et al.*, 1993a,b).

Neutralization assays

The human Fab fragments were assessed for their ability to reduce viral infectivity (HIV-1 MN or LAI) by a quantitative infectivity assay which enumerates multinucleate syncytia resulting from the fusion of infected CEM-SS cells with adjacent uninfected cells (Nara *et al.*, 1987), and a microplaque reduction assay using MT-2 cells as target cells (Hanson *et al.*, 1990). Virus stocks for both these assays were produced from chronically infected H9 cells. Selected Fabs were further tested for neutralization of HIV-1 infectivity by an envelope complementation assay assessing the ability of Fabs to inhibit a single round of viral infection (Helseth *et al.*, 1990). Viral stocks for this assay were generated by cotransfection of COS-1 cells by two plasmids, one expressing envelope glycoprotein, and the other expressing an envelope-deleted HIV-1 virus encoding chloramphenicol acetyl transferase as a reporter gene for infection of Jurkat cell targets. In all assays, controls without Fab and with the well-characterized neutralizing Fab b12 (Barbas *et al.*, 1992; Roben *et al.*, 1994) were run in parallel.

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